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Isolation, Selection and Identification of Polyaromatic Hydrocarbons (PAHs) Degrading Bacteria from Heavy Oil Waste (HOW)-Contaminated Soil

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ABSTRACT

The heavy oil waste (HOW) containing polyaromatic hydrocarbon (PAHs) is a persistent organic pollutants (POPs) that difficult to degrade. The new PAH degrading consortium was investigated from HOW contaminated soil in North Sumatera of Indonesia. The isolation, selection and identification of polyaromatic hydrocarbon degrading bacteria from soil contaminated by HOW was conducted to solve a bioremediation process. The isolation microbes from soil contaminated by HOW was performed using a minimum ONR7a media and followed on marine agar media for purification purposes. From the performed isolation results, 11 isolates were able to degrade PAHs compounds, such as phenanthrene, dibenzothiophene, or fluorene compounds. They grew at pH range of 4.8-8.2 and performed on emulsification activity in paraffin from 0.150-0.662. Three of them showed the best performance on HOW biodegradation capability and then successfully selected and identified as *Salipiger* sp., *Bacillus altitudinis*, and *Ochrobactrum anthropi*. using 16S rDNA. The HOW biodegradation as TPH-degradation were 38.66%, 59.60%, and 47.16%, respectively. Those isolated bacteria could potentially be as bioremediation agents to develop on bioremediation process for soils contaminated by HOW.

1. Introduction

Crude oil contains light and heavy oil fractions of total petroleum hydrocarbon (TPH). Heavy oil waste (HOW) contains polyaromatic hydrocarbons (PAHs). PAHs are relatively stable and recalcitrant in soil. Petrogenic PAHs are often marked as in abundance of alkyl substituted PAHs, such as alkyl naphthalenes, alkyl phenanthrenes, and alkyl dibenzothiophenes (Jong-Su *et al.* 2009). The content of hydrocarbons in the oil-polluted environment is relatively higher than the normal environment. In these circumstances, the survival microbes are that they capable to change the biochemical and molecular processes as an adaptive response to surrounding environmental changes. Little research in Indonesia was done to investigate potential bacteria for PAHs bioremediation. The bioremediation process of heavy oil waste could be done using a bacterial consortium that degrade hydrocarbon compounds. The high quantity of bacterial species capable of degrading hydrocarbons is possibly related to the universal availability of hydrocarbons in nature.

Mao *et al.* (2012) reported that bioremediation of a PAH-contaminated soil by a bacterial consortium enriched from the soil. One of petroleum degrading microbe sources, which have been widely explored, is an environment contaminated by crude oil waste. Isolates dominating in this environment consist of several genera, namely, *Alcaligenes, Arthrobacter, Acinetobacter, Nocardia, Achromobacter, Bacillus, Flavobacterium*, and *Pseudomonas*. Ma *et al.* (2010) isolated *Lysinibacillus sphaericus* as a PAH-degrading bacterium from the rhizospheres of vegetation in a contaminated field. The PAH degrading bacteria of *Pseudomonas, Marinobacter, Salinibacterium* and *Brevibacterium* were isolated from polluted sediment samples (Isaac *et al.* 2013). A variety of bacterial strains in *Acidovorax, Arthrobacter, Brevibacterium, Burkholderia, Comamonas, Mycobacterium, Pseudomonas,*

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and *Sphingomonas* can utilize phenanthrene as a sole carbon (Jong-Su *et al.* 2009). Several bacteria able to use fluorene, such as *Arthrobacter, Brevibacterium, Burkholderia*, and *Mycobacterium* (Jong-Su *et al.* 2009).

This study used HOW-containing PAHs, which are persistent organic compounds that are difficult to degrade by microorganisms. Only certain bacteria that can degrade polyaromatic hydrocarbons compounds existing in the heavy fraction of petroleum waste. Therefore, in this study, we performed isolation, selection, and identification of PAHs degrading bacteria from soil contaminated with heavy oil waste (HOW) in a petroleum industries in Indonesia as tropical countries. We investigated a new consortium bacteria that capable to use PAHs as sole sources of carbon and energy. The consortium bacteria was identified as PAH degrading bacteria to be potential for bioremediation process of HOW.

2. Materials and Methods

2.1. Soil Sampling

Soils contaminated by HOW were taken from crude oil contaminated soils, and the HOW was provided by an oil and gas company at North Sumatera, Indonesia. The samples were taken at the site of soil-contaminated areas, such as soil, sludge, and rhizospheres of grass. The contaminated soils were transported at low temperatures and then were prepared for enrichment culture within 2 days.

2.2. Enrichment Culture

Approximately 50 g of soil contaminated by HOW was added to 1 l of a general mineral media containing (mg/l): $FeSO_4 \cdot 7H_2O$ (200), $ZnSO_4 \cdot 7H_2O$ (10), $MnCl_2 \cdot 4H_2O$ (3), $CoCl_2 \cdot 6H_2O$ (20), $CuCl_2 \cdot 2H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (2), $Na_2MoO_4 \cdot 2H_2O$ (500), H_3BO_3 (30), and 5 ml of diesel oil (% v/v) as the carbon source. The use of diesel oil was for microbial consortium adaptation before using HOW. Enrichment cultures were conducted at room temperature (25-30°C) and were aerated for 2 weeks. The enrichment cultures were transferred to fresh media and 5% HOW was added as the carbon source. During enrichment cultures, the pH was maintained at pH 6-8 using H_2SO_4 8N or NaOH 6N. After several weeks, the HOW would be degraded by the consortium of bacteria.

2.3. Isolation of Hydrocarbon-degrading Bacteria

As much as 1 ml of the enrichment culture was diluted 10^5 times with saline solution (0.85% sterile NaCl) and then inoculated by the pour plate method using nutrient agar (NA) and marine agar (MA) from Difco. Each plate was labeled based on the medium type. The plates were incubated upside down at 30°C for one week. Observation of new colonies was performed every day. Each colony grown was coded then isolated by using minimum ONR7a medium and then PAH compounds (phenanthrene, dibenzothiophene, and fluorene) were added by using the sublimation technique. The ONR7a medium contained mineral medium that described by Syakti *et al.* (2013). Phenanthrene, dibenzothiophene, and fluorene were used as a contaminant model and then were enriched in the ONR7a medium as the sources of carbon and energy at a final concentration of 0.4 mg/l. The sublimation technique was conducted by heating phenanthrene, dibenzothiophene, and fluorene compounds. The evaporating PAH compounds would sublimate and were caught in ONR7a media given by cooling ice cubes. After finding the clear zone around the potential cultures, which were isolated, aseptically the evaporating PAH compounds were inoculated back into ONR7a media. Incubation was carried out at ambient temperature for 24-72 h. Then, refining was performed by using streak plates with MA media. The pure isolates were confirmed to ensure the selected culture's ability to degrade PAHs by subculturing into 5 ml of liquid medium containing 5 mg ONR7a of crystalline phenanthrene, dibenzothiophene, and fluorene compounds.

2.4. Selection of Bacterial Isolates

The obtained isolates were selected based on their ability to survive and grow on media containing HOW (5% in mineral media), as well as the ability to degrade heavy oil waste. Microbe selection was performed based on the ability to lower pH, to increase the microbial population and percentage of oil biodegradation in minimal media with carbon sources from HOW. All isolate cultures were incubated at 30°C for 3 weeks and the changing in number of population, pH, TPH (solid), and COD in slurry was observed after 0, 3, 7, 14, and 21 days. Two cultured replicates were taken and separated of the sludge (solid phase) and liquid phase. The solid phase was measured for oil content as total petroleum hydrocarbon (TPH) by solvent extraction methods (3540C EPA). The liquid phase was measured for population of bacteria using the TPC method, pH, and COD (Clesceri *et al.* 2005). The biodegradation of TPH was calculated from the results of TPH content of the slurry phase of HOW.

2.5. Biodegradation Test

The bacteria existing in HOW that potentially degrade PAH compounds were confirmed by the sublimation technique using minimum ONR7a media. The ability of bacteria to degrade PAHs was indicated by the clear zone on the phenanthrene compound and the discoloration on dibenzothiophene compounds. After finding the clear zone and discoloration around potential cultures that had been isolated, the potential cultures were

aseptically re-inoculated to ONR7a media. Incubation at ambient temperature was conducted for 24-72 h, and then the bacteria were transferred back to minimum ONR7a media. The purity of cultures were tested by planting in rich medium (Marine Agar medium, Difco). The pure isolates were subcultured into 5 ml of liquid ONR7a media containing 5 mg crystalline phenanthrene, dibenzothiophene and fluorene compounds. The confirmation test was conducted to ensure the ability of selected cultures to degrade PAHs.

2.6. Emulsification Activity Measurement Using Solid Paraffin

Emulsification activity was measured by using the method of Johnsons *et al.* (1992). The pure isolates were measured by using compact paraffin. A 4.5 ml supernatant was added to 0.5 ml hydrocarbon (solid paraffin). After mixing for 1 minute, they were incubated for 2 h and the emulsion stability was measured using the OD at 610 nm. The controls consisted of mineral water and hydrocarbons.

2.7. Identification of Selected Pure Cultures of Degrading Hydrocarbon

The selected isolates were identified molecularly (16s rDNA), as commonly reported. The molecular analysis performed included DNA extraction, PCR amplification, PCR product purification, and sequencing as follows.

DNA Extraction was performed by the Intragene Matrix kit (BioRad, Germany), followed by amplification. The PCR optimization results were obtained using primer 9F (5`-GAGTTTGATCCTGGCTCG-3') and 1510 R (5`- GGCTACCTTGTTACGACTT-3'). PCR was performed in a thermal cycler (T Shuzo) for 35 cycles. The initial step was at 94°C for 5 minutes, followed by 35 cycles consisting of 1 min denaturation at 94°C, 1 min annealing at 56°C, and 2 min extension at 72°C. After 35 cycles were completed, a final amplification for 4 min at 72°C, and cooling at 4°C for 30 min completed the reaction. The amplification results were fractionated by electrophoresis on a Mupid Mini Cell (Exu) 1% agarose gel in TEA buffer (Tris-EDTA) for 20 min at 100 V. The gels were incubated with ethidium bromide solution with a concentration of 1 ml/100 ml for 15 min. The results were visualized on a Gel Doc Printgraph (Bioinstrument, ATTO) using a UV transilluminator using the standard 100 bp DNA ladder (Promega) to determine the result and size of the DNA bands of the amplification results.

DNA purification of the PCR results was performed by precipitation. 0.1 and 2 times the total volume of 3 M Na-acetate pH 5.2 and 95% ethanol were added to the amplified PCR samples and then cooled at -20°C for 1 hour. Next, the samples were centrifuged at 10,000 × g for 30 min. The supernatant was discarded and the pellet was washed with 85% ethanol and then re-centrifuged at 10,000 × g for over 45 min. The supernatant was discarded and the pellet was precipitated at room temperature. The DNA pellet was dissolved with 15 µl of deionized water.

The next stage was cycle sequencing using the single primer, 9 F. The composition used for each tube was 1 ml 5 pmol primer, 150 ng purified DNA, 0.2 µl Big Dye Terminator sequences premix kit (Applied Biosystems), 2 ml of 5X buffer sequences and deionized water to volume of 10 ml. PCR amplification was then performed with a total of 30 cycles. The initial step was at 96°C for 20 sec, followed by cycles consisting of 10 sec denaturation at 96°C, annealing 5 sec at 50°C, and 4 min extension at 60°C.

Preparation was conducted by mixing 10 ml product cycle sequencing with 1 ml of 3 M Na-acetate and 25 µl absolute ethanol, vortexing and then allowed to stand for 15 min. The samples were then centrifuged at 16,000 × g for 20 min at room temperature. The supernatant was discarded and the pellet was washed with 70% ethanol and then re-centrifuged at 16,000 × g for 5 minutes. The supernatant was reset and the pellet was precipitated for 5 minutes. 15 µl Hidi-Formamide (Applied Biosystems) was added to the dried DNA pellet and was vortexed. The samples were then heated to 95°C for 2 min and immediately cooled on ice. In the next phase, the samples were injected to the sequencer ABI 3130 model (Applied Biosystems).

The BioEdit program was used for DNA analysis and blast in the NCBI data Library Gene Bank was performed. The phylogenetic analysis used multiple alignment programs of Clustal X version 1.83. The phylogenetic tree construction was based on genetic distances kinship with the neighbor joining method. The 16S sRNA sequences of three isolates were submitted to the European Nucleotide Archive (ENA); EMBL Nucleotide Sequence Database <datasubs@ebi.ac.uk> and registered at http://www.ebi.ac.uk/ena/data/view/LT708299-LT708301.

3. Results

3.1. Isolation of Hydrocarbon-degrading Bacteria

The result of enrichment culture appeared several kinds of PAH degrader (Tabel 1). Each isolated PAH degrader were tested to growth on media containing of phenanthrene (Phe), dibenzothiophene (Dbt), and fluorene (Flr) compounds as carbon sources. Emulsification activity was measured by using the method of Johnsons *et al.* (1992). The test results of the emulsification activity from the supernatants are shown in Table 1.

Table 1. Confirmation test of isolates using phenanthrene (Phe), dibenzothiophene (Dbt), and fluorene (Flr) compounds, and emulsification activity in paraffin

Isolate code	Growth oberserved in PAHs compounds			Emulsification activity (OD_{610})
	Phe	Dbt	Flr	
MY 1	+	-	-	0.463
MY 3	+	-	-	0.592
MY 5	-	+	-	0.465
MY 6	+	+	-	0.427
MY 7	+	+	-	0.662
MY 8	-	+	+	0.437
MY 9	-	+	-	0.150
MY10	-	+	-	0.286
MY11	-	+	-	0.421
MY12	-	+	-	0.189
MY13	-	-	+	0.318

3.2. The Growth of Selected Isolates

Figure 1 show the growth of isolated bacteria on mineral media containing 5% HOW. All isolates can degrade the HOW and well grow. The 3 of isolated bacteria were selected base on their growth characteristics and identified by 16S rDNA methods.

3.3. Identification of Hydrocarbon-degrading Bacteria

The 3 of 11 isolated bacteria were identified by 16S rDNA (Table 2) and the sequence of DNA were deposited at www.ebi.ac.uk. The phylogenetic neighborhood of the three isolated bacteria in a 16S rDNA gene sequence based tree is presented in Figure 2.

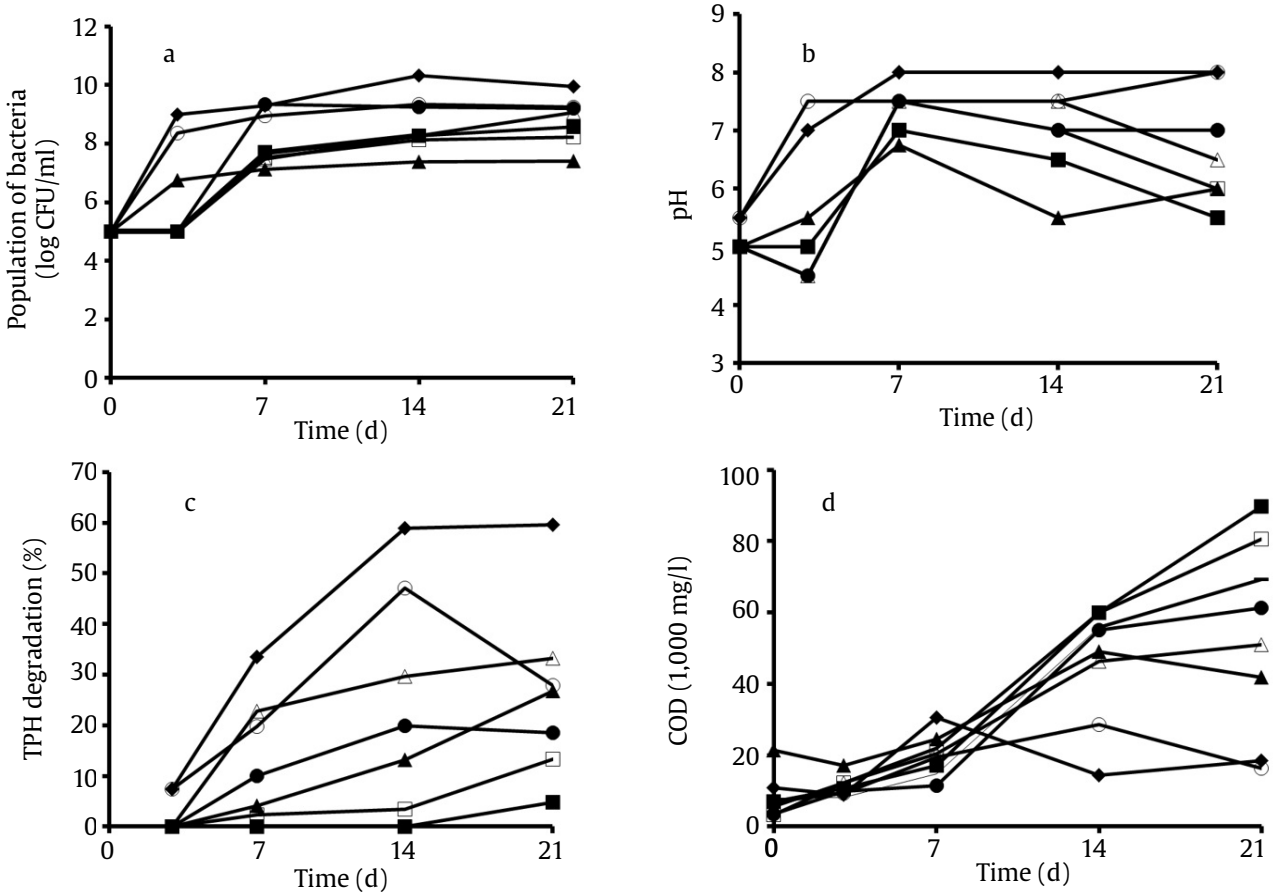

Figure 1. The growth of selected isolates: MY1 (■), MY3 (□), MY6 (▲), MY7 (Δ), MY8 (●), MY12 (♦), and MY13 (o) on 5% HOW; (a) number of population, (b) pH change, (c) TPH degradation, and (d) COD content in the slurry of HOW

Table 2. Identification molecular of 3 superior isolates

Isolate code	Assession number*	Length of 16S rDNA (bp)	Nearest bacteria taxon of BLAST	Homology (%)
MY7	LT708299	1027	*Salipiger* sp. PR55-4	100
MY12	LT708300	1241	*Bacillus altitudinis*	97
MY13	LT708301	1502	*Ochobactrum anthropi*	97

*http://www.ebi.ac.uk/ena/data/view/LT708299-LT708301

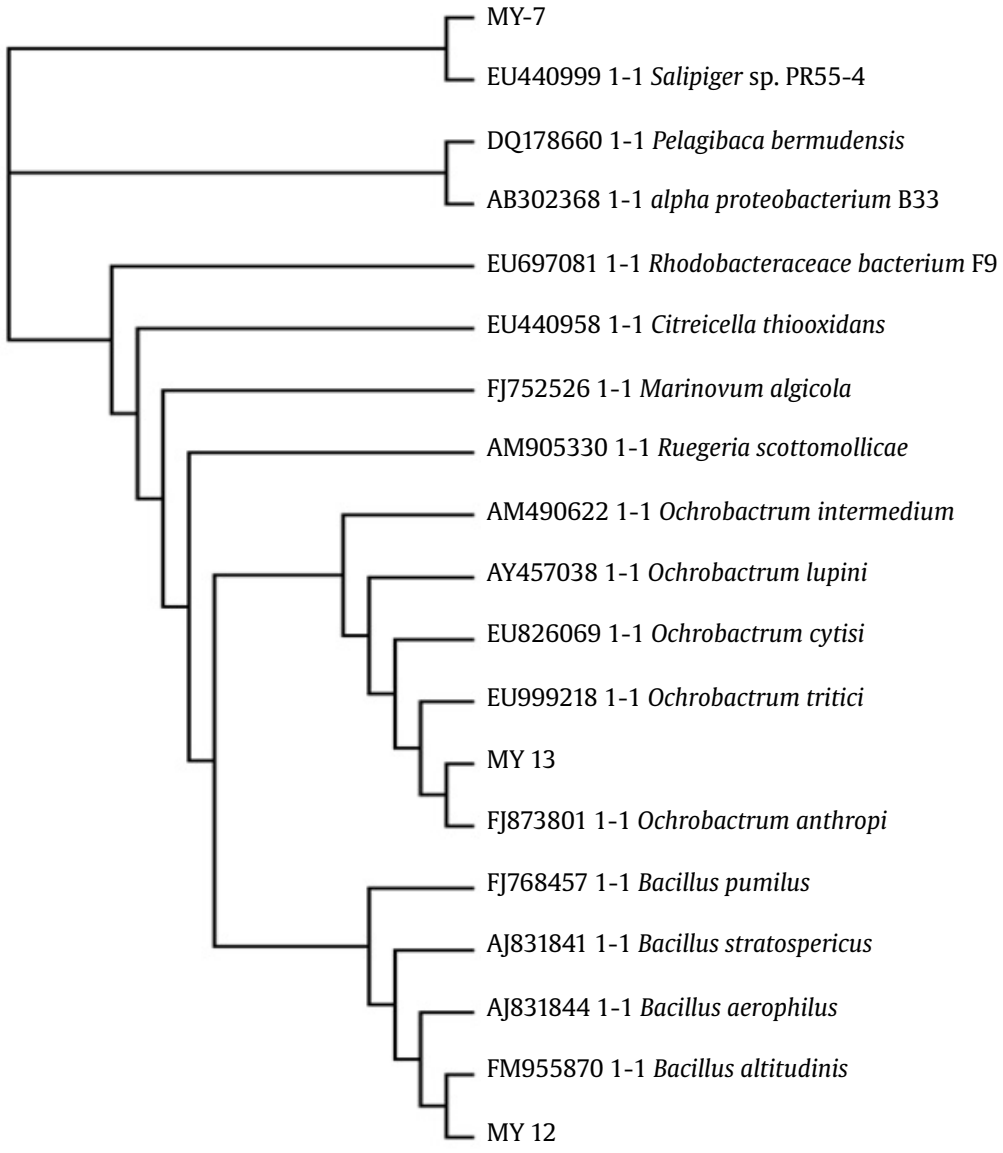

Figure 2. Phylogenetic neighborhood of three isolated bacteria in a 16S rDNA gene sequence based tree

4. Discussion

4.1. Isolation of Hydrocarbon-degrading Bacteria

The hydrocarbon-degrading bacteria were isolated from a bacterial consortium derived from soil contaminated by HOW. The population of bacteria from samples with the Total Plate Count (TPC) method was 1.12 x 10^8 CFU/ml. The cultured colonies are from consortium bacteria that are suggested to use PAHs as carbon sources. Charlena *et al.* (2010) reported that a consortium bacterium was used for bioaugmentation of soil contaminated by HOW by the land farming method. The consortium bacteria from heavy residual fuel oil (Beškoski *et al.* 2011) and PAH-contaminated soil (Mao *et al.* 2012) were reported as PAH degraders.

Through preliminary observation, we suggested that some colonies have a good affinity for PAHs (phenanthrene, dibenzothiophene, and fluorene), hence, their potential ability to degrade pollutants. The concentration of cells, which possibly enables degradation of hydrocarbons, was 1 x 10^6 CFU/g to 1 x 10^8 CFU/g. The bacteria existing in HOW that potentially could degrade PAH compounds were confirmed by the sublimation technique using minimum ONR7a media. The ability of bacteria to degrade PAHs was indicated by the clear zone of phenanthrene compound and discoloration of dibenzothiophene compounds. Of the colonies that appeared on the plates, 11 isolates were selected and confirmed to use PAHs (Table 1). Four isolates (MY1, MY3, MY6, and MY7) degraded phenanthrene (Phe). Eight isolates (MY5, MY6, MY7, MY8, MY9, MY10, MY11, and MY12) degraded dibenzothiophene (Dbt). Two isolates (MY8 and MY13) degraded fluorene (Flr).

4.2. Emulsification Activity Measurements Using Solid Paraffin

From the spectrophotometry results with a wavelength of 610 nm, all isolates produced surfactants that could emulsify solid paraffin and indicated the optical density from 0.150-0.662. The isolates of MY8 that degrade dibenzothiophene and fluorene showed an optical density at 0.437. While the isolate of MY13 that degrades fluorene had an optical density at 0.318. The isolates of MY7 had the ability to dissolve solid paraffin larger than the other isolates (0.662). The isolates of MY7 is consistent with the ability of this isolate, which also has the ability to degrade phenanthrene and dibenzothiophene. The emulsified organic compounds contained an intermediate product from paraffin degradation described by Cerniglia (1992).

4.3. Selection of Hydrocarbon Compound Degrading Bacteria Isolates

The ability of bacterial isolates to degrade hydrocarbons contained in heavy oil waste (HOW) was performed to select isolates, which would be used for further processing by observing isolates that had the best performance, which could be observed from the smallest TPH. By gas chromatography analysis, the chromatogram of HOW contained at least from C_6–C_{22} of alkane and polyaromatic compounds (data not shown).

After obtaining 11 isolates, the selection process was performed on the isolates having the ability to degrade dibenzothiophene compound i.e., isolates with codes MY5, MY9, MY10, MY11, and MY12. Of the 5 isolates, the MY12 isolate had a better ability to degrade hydrocarbons with TPH of 0.26%. The selection was conducted on the seven other isolates having the ability to degrade phenanthrene, dibenzothiophene, and fluorene compounds. Of the selection of isolates, MY1, MY3, MY6, MY7, MY8, MY12, and MY13, we selected 3 isolates with the best performance. The 7 isolates were incubated for 3 weeks and observed every week to measure the change in bacterial growth, pH media, TPH in soil/sediment, and COD of cultured media.

4.3.1. The Growth of Isolates

Figure 1a shows the growth of 11 isolates in 5% HOW. Three isolates, MY12, MY13, and MY6, demonstrated direct adaptation for growth, while others strains showed 3 days of the latent phase. The growth of MY8 is the same as MY12 after 7 days of inoculation. By day 21, the number of cells in each isolate continued to grow steadily. The number of cells in each single isolate was optimum at different times (Figure 1a). Isolates, MY1, MY7, and MY8, optimally grew on day 21, while other isolates optimally grew on day 14. The optimum growth of bacterial cells would increase the rate of degradation of hydrocarbons or would decrease the value of TPH in heavy oil waste.

Heavy oil waste contained toxic compounds with high concentrations so that isolates required a longer time to adapt to the environmental conditions. We observed MY12, which showed the best cell growth, since it had the highest number in population. Such argument was supported that the visualization of the growth of the isolates showed that the MY12 culture had more cloudiness in its medium, compared to the other isolates.

4.3.2. Changes in pH

The size of the population of bacteria was influenced by the environmental pH conditions. If the pH was in the normal range or neutral, the cell growth would grow well (Figure 1b). The optimum number of cells would enhance the ability of isolates in degrading hydrocarbons. The increase in the level of degradation was indicated from a decrease of the TPH value for each day. On day 0 and day 3, single isolates did not tend to demonstrate its ability to

lower TPH. This was caused by the low pH values on day 0 and 3 with values ranging between 4.5 and 6.5 (Figure 1b). The low pH values would cause stunted isolates. This could be seen from the population of bacteria on day 0 and day 3, the isolate cell growth was still very low. After 3 days, the pH value increased or decreased after the pH value was neutralized. If in the biodegradation process there was either an increase or decrease of pH, pH was controlled by adding NaOH or HCl until the pH became normal or neutral (pH 7).

4.3.3. TPH Degradation

The TPH value became a reference in determining the best three hydrocarbon-degrading isolates. The initial TPH of the slurry (solid) phase ranged from 17.2–23.2%. The more significant decrease in TPH indicated the better ability of isolates in degrading hydrocarbons in heavy oil waste. Based on the TPH degradation value, the best two isolates in the biodegradation process were MY7 and MY12 (Figure 1c). The highest degradation level of TPH by MY12 on day 21 was 59.60% (Figure 1c), with a final TPH value of 7.64% (w/w) of the initial TPH at 18.91% (w/w). The most drastic decrease in TPH occurred on day 14, and then there was a constant decrease in TPH. This was observed from the number of MY12 cells on day 12-14, which were the largest at 2.2 x 10^{10} CFU/ml. This showed that the greater number of cell isolates indicated the greater the TPH degradation occurred.

The second-best isolate was MY13 with a TPH degradation level of 38.66% (Figure 1c) with the final TPH value of 15.71% of the initial TPH of 24.43%. The higher the TPH degradation level, the better ability of isolates in degrading petroleum hydrocarbons. The TPH degradation level was optimum at different times. This was due to the different adaptability of each isolate.

Adaptability of isolates was demonstrated by the population of bacteria. The increase in the TPH value occurring in the biodegradation process was caused by the bacteria, which had passed through the stationary phase resulting in decreases not only in the number of bacterial cells, but also the ability of degrading hydrocarbons. The anomalous degradation level of TPH occurred in MY12 because the hydrocarbon content in HOW was uneven, so the difference in total hydrocarbons caused a negative value.

4.3.4. COD Value

The emulsified organic compounds and intermediate PAH degradation products in cultured isolates were measured as chemical organic compounds (COD). Figure 1d shows the COD value in liquid media where it represented the organic compounds released from soil contaminated by HOW. The biosurfactant produced by bacteria will enhance to disperse or release the organic compound from soil matrices to the liquid phase. The COD value increased during the incubation period, however after 14-21 days, the COD value fluctuated in accordance to bacterial activity on degradation and mineralization to simple products. The MY12 isolate had the lowest COD value compared with other single isolates (Figure 1d). The low COD value indicated that the organic compounds contained in the sample (liquid phase) were fewer; this was in line with the highest in TPH degradation (Figure 1c) by the MY12 isolate. From the observed parameters, isolates, MY7, MY12, and MY13, had a better ability to degrade heavy oil waste (Figure 1c). These three isolates were further identified to determine the bacterial species contained in each.

4.4. Identification of Isolates MY7, MY12 and MY13

The identification of bacteria was performed molecularly, based on genetic analysis, part of the 16S ribosomal bacterial DNA. Isolation of DNA was preceded by growing bacterial isolates in Marine agar media and then incubating for 72 h. Bacterial DNA extraction was conducted by using the GES method (Picher *et al.* 1989).

PCR amplification included Primer20 F (5'-GATTTTGATCCTGGCTCAG–3') and 1500 R (5-GTTACCTTGTTACGACTT–3'). The PEG *precipitation method* was performed on the purified PCR samples (Hiraishi *et al.* 1995) and then followed by cycle sequencing with primers 520 F (5'-GTGCCAGCAGCCGCGG-3'), and 920 R (5'-GTCAATTCCTTTGATTT-3'). The resulting cycle sequencing samples were purified again with the ethanol *purification method*. The reading analysis of nitrogen base sequences was performed with an automated DNA *sequencer* (ABI PRISM 3130 *Genetic Analyzer*).

The raw data in the subsequent sequencing results were processed in *Trimming* with the program, MEGA 4, and *assembling* with the BioEdit program and

further converted to the fasta format. The results of DNA sequencing in fasta format were then processed in blast to search for homology in the DNA data base center in the DDBJ (http://www.ddbj.nig.ac.jp). Table 2 shows the results of the molecular identification of the 3 superior isolates and Figure 2 shows the phylogenetic neighborhood of the three isolated bacteria by a 16S rDNA gene sequence based tree.

The MY7 isolate (accession no. LT708299) is 100% homologous to *Salipiger* sp. PR55-4 (accession no. EU440999) (Table 2 and Figure 2). This result showed high sequence similarity to *Pelagibaca bermudensis* (DO178660), *Alpha proteobacterium* B33 (AB302368), and *Rhodobacteraceae bacterium* F9 (EU69708), respectively. The *Salipiger* sp. PR55-4 is gram-negative and rod-shaped, is included in *chemoheterotrophic aerobic bacteria* (cannot grow under anaerobic conditions), has resulting *catalase* and *phosphatase* enzymes, and does not produce acid from *carbohydrates*; they are also not able to grow with *carbohydrates* or amino acids as a sole source of carbon and energy. *Salipiger mucescens* is a gram-negative rod isolated from a hypersaline habitat. This species is chemoheterotrophic and strictly aerobic, moderately halophilic, and is exopolysaccharide producing (Martı´nez-Ca´ novas *et al.* 2004).

From the confirmatory test performed, the MY7 *Salipiger* sp. can degrade phenanthrene and dibenzothiophene (Table 1), optimum growth is at pH 5-8 (Figure 1b), and HOW degradation reached 33.19% at 21 days (Figure 1c). The isolates of MY7 *Salipiger* sp. can use sole carbon as a source from palm oil, kerosene, diesel oil, and HOW (data not shown). This bacterium had similar growth to others isolated by some authors. *Salipiger nanhaiensis* was isolated from the deep-sea water in China (Dai *et al.* 2015). Related cross phylogenicity of *Roseobacter* is *Roseovarius* sp., that is coco-shaped, forms small raised creamy colonies on nutrient agar medium, and can degrade <15% of phenanthrene after 10 days. The maximum biodegradation efficiency of 28.4% was obtained at pH=8.2, temperature ≈35°C, salinity=30 ppt, NH_4Cl=0.13 g/l and inoculum size=0.2 OD 600 nm. The abiotic elimination of phenanthrene during bio¬degradation experiments was negligible (Shahriari *et al.* 2013).

The isolate of MY12 (accession no. LT708300) is 97% homologous to *Bacillus altitudinis* (FM955870) (Table 2 and Figure 2). This result showed high sequence similarity to *Bacillus aerophilus* (AJ831844), *Bacillus stratosphericus* (AJ831841), and *Bacillus pumilus* (FJ768457), respectively. From the confirmatory test performed, the *Bacillus altitudinis* MY12 bacteria can degrade dibenzothiophene (Table 1), optimum growth is at pH 5.5-8.2 (Figure 1b), and the highest of HOW degradation reached 59.6% at 21 days (Figure 1c). The isolates of MY12 *Bacillus altitudinis* can use sole carbon as a source from palm oil, kerosene, diesel oil, and HOW (data not shown). *Bacillus altitudinis* are rod-shaped, gram-positive bacteria (Shivaji *et al.* 2006), which can only degrade dibenzothiophene. *B. pumilus* has a kinship with *B. altitudinis*, having the ability to degrade *naphthalene* compounds found in petroleum waste (Calvo *et al.* 2004). *Bacillus altitudinis* DHN8 isolated from a compost pit was assessed for xylanase production by utilizing a sorghum straw (Adhyaru *et al.* 2014). The bacterium, *Bacillus altitudinis* AP-MSU, is able to produce esterase and was isolated from the gut of the marine fish, *Sardinella longiceps* (Esakkiraj *et al.* 2012).

The isolate of MY13 (accession no. LT708301) is 97% homologous to *Ochrobactrum anthropi* (FJ873801) (Table 2 and Figure 2). This result showed high sequence similarity to *Ochrobactrum cytisi* (EU826069), *Ochrobactrum tritici* (EU999218), *Ochrobactrum lupini* (AY457038), and *Ochrobactrum intermedium* (AM 490622). From the confirmatory test performed, the *Ochrobactrum anthropi* MY13 bacteria can degrade fluorene (Table 1), has an optimum growth at pH 5.5-8.2 (Figure 1b), and the highest of HOW degradation reached 47.16% at 14 days (Figure 1c). The isolates of MY13 *Ochrobactrum anthropi* can use sole carbon as a source from diesel oil and heavy oil waste (HOW), but it cannot grow in media containing palm oil and kerosene (data not shown). The isolate of *Ochrobactrum anthropi* MY13 will be utilized in future studies.

The genus, *Ochrobactrum*, belongs to the family, *Brucellaceae*, within the alphaproteobacterial order, Rhizobiales. *Ochrobactrum* strains were isolated from diverse habitats including soil, plants and their rhizospheres, wastewater, animals and humans (Bathe *et al.* 2006). This is the first finding of *Ochrobactrum anthropi* MY13 in soil contaminated by HOW. In medical science, the first reported case was in Malaysia of a catheter-related bloodstream infection caused by *Ochrobactrum anthropi* in a patient with underlying diabetes and ESRF (Rohani and Tzar 2013). Seven methyl parathion-degrading bacteria were isolated from long-term methyl parathion-

contaminated soil and were found as one of genera to belong to *Ochrobactrum* (Zhang *et al.* 2005).

Ochrobactrum strains are of particular interest for bioremediation (Bathe *et al.* 2006). They are capable of degrading organophosphorus pesticides, such as parathion and methyl parathion (Zhang *et al.* 2005), petroleum waste (Katsivela *et al.* 2005), fungicide chlorothalonil (Kim *et al.* 2004), phenol (El-Sayed *et al.* 2003), and the toxic organic solvent dimethylformamide (DMF) (Veeranagouda *et al.* 2006). *Ochrobactrum anthropi* YD50.2. denitrified NO_2− or NO_3−. The gene clusters for denitrification (*nar, nir, nor*, and *nos*) were cloned from *O. anthropi* YD50.2, in which the *nir* and *nor* operons were linked (Doi *et al.* 2009). These results have generated considerable interest in the application of *O. anthropi* as a bioremediation agent.

Many researchers isolated the petrophylic hydrocarbon degraders from different environments. The petrophylic hydrocarbon degraders spread throughout the world-wide environment from Asia, Europe, America and Africa, which are isolated from soil-contaminated areas, hypersaline habitats, deep sea water, polluted sediments, mangrove sediments, agricultural soils, etc. (Table 3). We reported that the petrophylic consortium bacteria were successfully isolated and identified from soil contaminated by heavy oil waste (HOW) that contain polyaromatic hydrocarbon compounds in Indonesia.

4.5. Future Applications

The soil or sediment contaminated by crude oil or bioremedied soil contains a heavy (C_{24}-C_{40}) fraction of TPH. Rosenberg and Ron (1998) described the bioremediation of petroleum contamination by several methods, such as land farming. The oil and gas company that operates in Indonesia generally treat the crude oil-contaminated soil by land farming and biostimulation of indigenous consortium microorganisms with fertilizer (N, P, K). These bioremediation methods achieve the high biodegradation rate at several weeks, then slow down for several months. At the end of the land farming process, the bioremedied soil reaches a residual TPH between 2-4%, and then it becomes very difficult to decrease to comply to the Indonesian regulation at 1% TPH. The bioremedied soil is further treated by phytoremediation to degrade the residual TPH and heavy metals. Arifudin *et al.* (2016) reported that bioremediation of crude oil-contaminated soil by consortium bacteria decreased the TPH from 4.22% to approximately 1%. After analyzing the bioremedied soil by chromatography GCMS, the chromatogram shows that high molecular weights (>C17) dominated the PAH.

Many researchers are interested in contributing to findings of PAH degraders from many sources to treat sediment and soil contaminated by PAH (Table 3). Cerniglia (1992) reported three main pathways for PAH degradation by fungi and bacteria. Those bacteria are indigenous consortium microbes, because the consortium bacteria will work to degrade or oxidize the target compounds by extracellular enzymes.

Beškoski *et al.* (2011) reported that the mazut (heavy residual fuel oil)-polluted soil was exposed to bioremediation in an *ex situ* field-scale study by biostimulation and bioaugmentation with microbial consortia. The reductions for the aliphatic, aromatic, and nitrogen–sulfur–oxygen and asphaltene fractions are 96%, 97%, and 83%, respectively. However, the isoprenoids, pristane and phytane, were more than 55% biodegraded. Mao *et al.* (2012) reported that bioremediation of PAH-contaminated soil (90.6% of which were 4- and 5-ring PAHs) was carried out for 56 days and showed at 35.8% of total PAHs were removed from the soil with the addition 20% of a bacterial consortium suspension.

This research shows that the indigenous isolated bacterial play a role in PAH biodegradation of HOW. The three new isolates of *Salipiger* sp. MY7, *Bacillus altitudinis* MY12, and *Ochrobactrum anthropi* MY13 were isolated from soil contaminated by HOW. They can degrade PAH compounds of phenanthrene, dibenzothiophene, and fluorene. The mixed culture of three isolates may synergistically work on biodegradation of heavy oil waste. This bacterial consortium may be a promising agent for bioremediation of PAH-contaminated soils, such as heavy oil waste (HOW). The bioaugmentation of PAH degraders by the bioremediation method (land farming or biopile) can enhance TPH biodegradation and can reduce the residual PAH in bioremedied soil. The mixed culture could enhance the biodegradation of soil contaminated by HOW.

5. Conclusion

From the heavy oil waste (HOW) contaminated soil, we successfully investigated the consortium bacteria, which were capable of degrading phenanthrene,

Table 3. PAH degraders isolated from different environment worldwide

Sites	Country	Isolates	Compounds	References
Soil contaminated by heavy oil waste (HOW)	Indonesia	*Consortium bacteria: Bacillus altitudinis, Salipiger* sp. PR55-4, *Ochrobactrum anthropi*	PAHs, phenanthrene, dibenzothiophene, and fluorene	This study
Mazut (heavy residual fuel oil)-polluted soil	Serbia	*Consortium bacteria*	PAH, the aliphatic, aromatic, and NSO-asphaltene fractions, pristane and phytane	Beškoski *et al.* (2011)
PAH-contaminated soil	China	*Consortium bacteria*	PAHs	Mao *et al.* (2012)
Contaminated area	United Kingdom	*Fungi* and *bacteria*	PAHs	Bamforth and Singleton (2005)
Sediment contaminated by fuel	Singapore	*Consortium bacteria sediment*	PAHs, naphthalene, acenaphthene and phenanthrene	Sherafatmand and Ng (2015)
Hypersaline habitat in Murcia	Spain	*Salipiger mucescens*	-	Martı´nez Ca´ novas *et al.* (2004)
Hypersaline habitat in Murcia	Spain	*Salipiger mucosus*	Genomic study	Riedel *et al.* (2014)
Deep sea water	China	*Salipiger nanhaiensis* sp. nov.	-	Dai *et al.* (2015)
Mangrove surface sediment	India	*Roseovarius* sp.	PAHs, phenanthrene	Shahriari *et al.* (2013)
Sediment of crooked lake	Indiana, USA	*Mycobacterium flavescens, Rhodococcus* sp.	Pyrene	Dean-Rose (2003)
Mangrove, Huian	China	*Bacillus megaterium, Bacillus cereus*	Fluorene, phenanthrene, fluoranthene, and pyrene	Lin and Chai (2008)
Soil	Korean	*Acidovorax, Arthrobacter, Brevibacterium,Burkholderia, Comamonas, Mycobacterium, Pseudomonas,* and *Sphingomonas*	Phenanthrene, fluorene	Jong-Su *et al.* (2009)
Soil	Qatar	*Pseudomonas geniculata* and *Achromobacter xylosoxidans*	PAHs	Al-Thani et al.(2009)
Soil samples at a crop garden in Wuxi	China	Bacillus subtilis FQ06, *Caulobacter* sp. 3-3, *Bacillus pumilus* NAPCC-1, *Bacillus* sp. CAt2NG, *Stenotrophomonas maltophilia, Labrys* sp. LLQ-6, *Rhizobium* sp. D255c, *Burkholderia* sp. lxb-13, *Cytophaga* sp. SSL03	PAHs, phenanthrene, pyrene, benzo[a]pyrene	Ma *et al.* (2010)
Polluted sediment samples of Caleta Cordova harbor	Argentina	*Pseudomonas, Marinobacter, Salinibacterium,* and *Brevibacterium*	PAHs	Isaac *et al.* (2013)
Apple and pear fruit	Egypt	*Bacillus altitudinis, Bacillus pumilus*	Pathogenic of fruit	Elbanna *et al.* (2014)
Mangrove sediments in Hong Kong, South China	China	*Sphingomonas, Microbacterium, Rhodococcus*	PAHs (fluorene, phenanthrene, fluoranthene and pyrene)	Li *et al.* (2009)

Table 3. Continued

Sites	Country	Isolates	Compounds	References
Landfarmed soil	Greece	Genera of *Enterobacter, Ochrobactrum,* and *Alcaligenes*	Petroleum waste	Katsivela *et al.* (2005)
-	South Korea	*Ochrobactrum anthropi* SH35B	fungicide chlorothalonil	Kim *et al.* (2004)
Agricultural soil and on the wheat rhizoplane	Germany	*Ochrobactrum anthropi, Ochrobactrum intermedium, Ochrobactrum tritici* and *Ochrobactrum grignonense*	-	Bathe *et al.* (2006)
Marine	China	*Onchrobactrum sp.* BAP5	benzo[a]pyrene	Yirui *et al.* (2009)
Bloodstream	Malaysia	*Ochrobactrum anthropi*	-	Rohani and Tzar (2013)
Soil polluted with petroleum oil	Egypt	*Achromobacter xylosoxidans, B. Amyloliquifaciens,*	Pyrene	Abo-State *et al.* (2013)

dibenzothiophene, and fluorene compounds. From 11 isolates observed, the bacteria having the best performance of three in TPH degradation, emulsification and PAHs degradation are *Salipiger* sp. MY7, *Bacillus altitudinis* MY12, and *Ochrobactrum anthropi* MY13. The three isolated bacteria could be potentially applied as bioremediation agents of heavy oil waste that contains PAHs.

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